

NBSIR 74-607(R)

QUARTERLY REPORT  
FDA CONTRACT NO. 74-58(0)

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R. Schaffer

Analytical Chemistry Division  
Institute for Materials Research  
National Bureau of Standards  
Washington, D.C. 20234

February 1975

Interim Report for Period  
October--December 1974

Prepared for  
Bureau of Medical Devices and  
Diagnostic Products  
Food and Drug Administration  
Rockville, Maryland 20852



Quarterly Report October--December 1974  
to the  
Food and Drug Administration  
by the  
National Bureau of Standards

Progress is reported toward the goal of establishing the accuracy of several clinical reference methods, namely, those for glucose, sodium, potassium, lithium, magnesium, and chloride in serum, for lead in blood, and for uric acid. The work performed during the interval October-December 1974 is reviewed, method by method.

TASK 1a: SERUM GLUCOSE

Base-line Method

Glucose analysis by the method of isotope-dilution mass spectrometry (ID-MS) has begun. Samples (see Quarterly Report April-June 1974) being studied are 1) known mixtures of the 1,2:5,6-di-O-isopropylidene derivatives of glucose-6,6-d<sub>2</sub> (dideuteroglucose) or U-<sup>13</sup>C-glucose (uniformly labeled <sup>13</sup>C-glucose) and natural glucose to determine the accuracy of recovering by MS the known proportions of the isotopic molecules in the molecular form measured; 2) known mixtures of either labeled glucose with natural glucose; these mixtures of the isotopic and the natural glucose are converted into the diisopropylidene derivative, and then the proportions are measured by MS to determine whether changes in the proportions of the labeled to unlabeled forms might occur on synthesizing and separating the derivative; and 3) multiple portions of a single serum containing glucose to which different proportions of either one of the labeled glucoses are added and then the derivative is synthesized, isolated and tested by MS to assay for the glucose content of the serum.



The results from 1) and 2) provide estimates of attainable accuracy. The results from 3) provide an estimate of precision. The overall result that has emerged from analyses performed thus far is that the ID-MS approach will afford base-line accuracy and precision within 1% for the glucose content of serum.

The receipt and installation of the improved multiple ion selection unit for the mass spectrometer (referred to in Quarterly Report July-September 1974) are expected momentarily.

#### Analytical Work

Second round results obtained by use of the candidate reference method (hexokinase) were reviewed with participants at a meeting held in November 1974.

For this second round in addition to analyzing for glucose in serum samples, participating laboratories were asked to report the spectrophotometric absorbances of a set of aqueous solutions of acid dichromate that were supplied; these measurements were sought to enable the committee to evaluate the influence of the spectrophotometers being used on the slopes of the glucose calibration curves obtained in the individual laboratories. (In the first round it was found that these calibration curves differed from lab to lab.) The additional spectrophotometric data were considered statistically by examining 1) the slopes of the lines obtained by correlating each laboratory's reported dichromate absorbance values against those measured by NBS and 2) the slopes of the lines connecting the set of absorbances measured in each laboratory in setting up its glucose calibration curve. Despite the small range of values over which correlations were calculated, it could be concluded that the differences between the slopes of the glucose calibration curves are reflected by the corresponding differences in slopes measured



on the dichromate solutions; hence, the characteristics of the different spectrophotometers largely determine the differences observed in glucose calibration curves.

The serum samples were analyzed in the second round in two separate ways: 1) by the previous protocol using manual pipetting and, where laboratories were appropriately equipped, 2) by an alternative protocol in which an automatic pipetter, whose precision could meet necessary specifications, was employed.

The results obtained by the method employing manual pipetting only are as follows:

Average mg/dl	Within Lab CV %	Between Lab CV (Single Test/Lab) %	Between Lab CV (4 Test Avg/lab) %
40.4	1.49	5.71	5.56
75.4	1.05	2.66	2.50
134.3	1.89	2.10	1.32
205.9	1.44	1.65	1.08
450.0	0.62	1.33	1.22

Results for the method employing the automatic pipettor are as follows:

Average mg/dl	Within Lab CV %	Between Lab CV (Single test/lab) %	Between Lab CV (4 Test Avg/lab) %
40.8	1.91	3.60	3.20
76.0	1.02	1.90	1.68
133.9	1.13	1.43	1.04
204.1	0.70	1.04	0.85
450.3	0.52	0.92	0.80

The second round results reveal a measureable improvement in the between-laboratory coefficient of variation (CV) over the results from the first round. However, the results continue to show a high between-laboratory coefficient of variation for samples containing glucose at the lowest concen-





tration levels examined.

Although only about one-half of the number of laboratories that performed the manual method also performed the method using the automatic pipettor, the results from the latter method clearly reveal better between-laboratory coefficients of variation over results from the manual procedure.

#### TASK 1b: SODIUM POTASSIUM, LITHIUM, MAGNESIUM AND CHLORIDE

##### Base-line Methods

Application of the base-line methods for these electrolytes is contingent on NBS receiving serum samples from the CDC. NBS has contracted for the needed bulk supply of serum and its delivery to CDC where the serum is chemically treated and vialled as samples for the study of the reference methods for each of the electrolytes. A minor technical difficulty in processing the serum has slowed the production of these samples. As a consequence at this time, only the samples for the lithium study have been received, and those having magnesium are expected within the next week or so. Sodium and potassium samples are expected by early March and chloride samples will follow one month later. Base-line methods are to be performed on these samples as they are received.

##### Lithium

The protocol for the candidate lithium reference method is appended to this report. The first test round of the protocol for lithium has been carried out but NBS base-line values are not yet available; hence, the results received from the participating laboratories are summarized only in terms of precision:

<u>Sample Number</u>	<u>Average mmol/l</u>	<u>Standard Deviation mmol/l</u>	<u>CV %</u>
1	1.005	0.023	2.32
2	1.497	0.030	1.98
3	1.975	0.031	1.57



## Magnesium

The protocol for this reference method has been prepared following the format of the lithium protocol. Participating laboratories are about to be supplied with the protocol, and with magnesium gluconate to use as the standard and serum samples to use for practice runs. As soon as the magnesium serum samples are received from CDC, they will be sent to the laboratories for the first round of study to begin.

## Sodium-Potassium and Chloride

The writing of candidate protocols for each of these electrolytes is near completion. Analytical work awaits the arrival of samples.

### TASK 1c: LEAD IN BLOOD

An extraction-atomic absorption procedure for determining lead in blood was used by six participating labs as a first round of study of a protocol for the reference method. A meeting to review the results of that round of study and to criticize the protocol was held in November 1974. The best reported results (from only 2 of the participants) were within 20% of the ID-MS target values; however, three labs reported values that were off by more than 50%. Possible sources of difficulty and ambiguity and recommended changes in the protocol were discussed for use in a revised protocol. A highly detailed protocol has been prepared. Further detail may need to be added to the protocol, but this will become obvious on consideration of the results from the next round of study.

Because of the uncertainty of the accuracy of analyses for lead by methods other than ID-MS, samples to be used for later rounds of study are to be analyzed by the NBS ID-MS method before being sent to participants.

To ensure freedom from lead contamination, all materials that come into contact with the blood samples (syringes, extraction tubes, Teflon tape, etc.) are being tested by ID-MS. ID-MS values are now being obtained on the samples for round two.



## TASK 1d: URIC ACID

### Base-line Method

The ID-MS approach to definitive values for uric acid in serum is being pursued. Because uric acid is present in serum at concentrations of about 0.1 mg/ml, we expect to use gas chromatography in the last stage of purification of the mixture of isotopic molecules. However, uric acid is insufficiently stable to be passed through a gas chromatograph to the MS. Hence, a volatile derivative of uric acid is needed. (This is somewhat like the situation with glucose, for which the diisopropylidene derivative was needed to provide sufficient volatility.)

Methylation has been used to obtain volatile derivatives, but a number of different derivatives are produced. Now we find the same situation on use of ethylation procedures. Ethylation would be preferable as it may avoid possible difficulty with interferences due to methylated substances (e.g., drugs) that may be present in serum samples.

An alternative route to the needed volatile derivative is acylation. Such reactions of uric acid are poorly understood. However, our examination of such methods has led to the isolation of a possibly useful derivative having isobutyryl residues. The structure of this product is being studied.



Expenditures October--December 31, 1974  
(from NBS Accounts Division Statement)

Labor 2283 hours

Labor, leave, etc.	\$68,245.00
Other objects	<u>53,200.00</u>
	\$121,445.00





## PROTOCOL:

### The Determination of Lithium in Serum

#### I. Scope

The method is designed to provide accurate values of the lithium concentration in serum or aqueous solutions as submitted for analysis, provided strict adherence to this protocol is maintained. The method does not assure adequacy or integrity of sampling *per se*. The method has applicability, at the specified accuracy for lithium, in the range from 0.5 to 2.5 mmol/l and is an adaptation of a procedure by Pybus and Bowers [1].

#### II. Reagent Specifications

1. At the time of preparation, distilled and/or deionized water should exhibit a specific resistance of at least 1.0 Mohm cm at  $25 \pm 2$  °C. At the time of use, aspiration of this reagent grade water should show no detectable signal under the instrumental settings used for this analysis. The water should be available in large quantity for use as a diluent and for the final rinse operation on all glassware and apparatus coming in contact with the solutions involved.
2. Lithium standard solutions are to be prepared from Standard Reference Material  $\text{Li}_2\text{CO}_3$  (SRM 924) [2] issued and certified by the National Bureau of Standards (NBS). The SRM  $\text{Li}_2\text{CO}_3$  should be stored at room temperature in a desiccator containing silica gel or equivalent. (NOTE: The lithium in the SRM is depleted in the  $^6\text{Li}$  isotope; thus the molecular weight for this  $\text{Li}_2\text{CO}_3$  is 73.94855, and calculations should be based on this value.)
3. Sodium and potassium chlorides, hydrochloric and nitric acids, and 95% ethanol (V:V) meeting ACS [3] (or equivalent)



specifications are to be used.

4. Dilute nitric acid is prepared by making a twenty-fold dilution of concentrated  $\text{HNO}_3$  (15.4 M) with water.
5. Dilute hydrochloric acid is prepared by making a five-fold dilution of concentrated  $\text{HCl}$  (11.6 M) with diluent solution (see IV-1). Prepare approximately 25 ml.

### III. Glassware Specifications

All volumetric glassware (pipets and volumetric flasks) should meet NBS Class A specifications [4] (or equivalent).

All glass or plastic surfaces that come into contact with reagents, water, diluent, or sample must have been previously cleaned as follows (see also: Section V-3):

1. Use routine cleaning procedure (warm water with detergents, plus usual rinses)
2. Soak glassware for 30 min in dilute  $\text{HNO}_3$ , (NOTE: Concentrated  $\text{HNO}_3$  will etch glass and produce sites for ion adsorption).
3. Rinse six times with a volume of water equal to or greater than 10% of the container volume and
4. Use apparatus immediately or air dry (inverted) in a dust-free environment for future use.

### IV. Preparation of Reagents

1. Diluent (Blank) Solution: Contains  $\text{NaCl}$  at 140 mmol/l and  $\text{KCl}$  at 5.0 mmol/l. Add 16.36 g of  $\text{NaCl}$  and 0.746 g of  $\text{KCl}$  to a 2-liter volumetric flask, dissolve in water, and add water to below the calibration mark. At  $25 \pm 2^\circ\text{C}$ , fill to the calibrated volume with water. Mix by inverting the flask 30 times.
2. Lithium Standard Stock Solution: Weigh accurately to the nearest one tenth milligram 0.3698 g of  $\text{Li}_2\text{CO}_3$  (NBS SRM 924) and transfer it quantitatively to a 1-liter volumetric flask.



To dissolve the  $\text{Li}_2\text{CO}_3$ , just cover the bottom of the flask with diluent solution and slowly add 10 ml of dilute HCl (See Section II-5). Fill to the mark at  $25 \pm 2$  °C using diluent solution to give the 10.0016 mmol/l lithium standard stock solution.

3. Lithium Standard Diluted Solutions: Transfer 5-, 10-, 15-, 20-, 25-, and 30-ml aliquots of the lithium standard stock solution to six 100 ml volumetric flasks in that order. Fill each to the mark at  $25 \pm 2$  °C using diluent solution to give the diluted standards of 0.50-, 1.00-, 1.50-, 2.00-, 2.50-, and 3.00- mmol/l of Li, respectively. (NOTE: The concentrations of the lithium standard diluted solutions must be calculated and reported to four decimal places as in Table 1, Section VI-4).

#### V. Procedure for Pipetting and Diluting

All solutions should be at  $25 \pm 2$  °C.

All blank, diluted standard and unknown sample solutions are diluted ten-fold using a 5-ml Class A volumetric pipet in the "to contain" mode and 50-ml volumetric flasks. Only one volumetric pipet is used throughout to avoid errors that would be caused by differences in drainage between the aqueous or dilute acid and serum solutions, using different pipets. If 50 ml of a standard is insufficient for the total test, prepare a second 50-ml portion.

##### 1. Pipetting and Diluting, General:

Transfer approximately 15 ml of water to a 50-ml volumetric flask and then add 5 ml of the appropriate solution by the following procedure. Fill the 5 ml Class A pipet to approximately 1.0 cm above its calibration mark. Place the index finger over the top end of the vertical pipet, contact the tip of the pipet with the side of the container and deliver excess solution until the meniscus is at the calibrated mark on the pipet. Remove the pipet from



contact with the container and tilt the pipet to an approximately horizontal position. Then remove excess liquid from the exterior of the delivery end of the pipet by wiping with clean absorbent paper. Direct the delivery end of the pipet into the receiver while bringing the pipet back to a vertical position. The sample should be delivered by contacting the pipet tip with the receiver wall and allowing the pipet to drain. After drainage of the pipet stops, gently blow out the residual liquid. Rinse the pipet tip into the volumetric flask with 4 ml of water delivered, for example, from a disposable Pasteur or similar pipet. (Caution: New pipets need to be cleaned.) Rinse the 5-ml volumetric pipet three times by filling with water, each time delivering the contents into the receiver by drainage against the inner wall of the flask above the liquid level. Then fill to the calibrated volume with water at  $25 \pm 2$  °C and mix thoroughly with 30 inversions.

2. Pipetting and Diluting, Working Blank and Lithium Standard Working Solutions:

Prepare the working blank and the 0.50-, 1.00-, 1.50-, 2.00-, 2.50-, and 3.00-mmol/l lithium standard working solutions in that order. Fill the 5-ml pipet with the diluent solution (blank) or lithium standard diluted solution to be transferred and discard that portion of the solution. Repeat twice. Then fill the pipet, adjust to volume, deliver to the receiver flask, rinse the pipet, dilute using water and mix as described in Section V-1.

3. Pipetting and Diluting Sera (The volume of the serum specimens provided is insufficient for use of the pipet-conditioning technique described in Section V-2; hence, the pipet must be cleaned and dried for each serum sample.):

Fill the clean, dry 5-ml pipet with the serum sample,





- 5 -

adjust to volume, deliver and rinse into the receiver, and dilute as described in Section V-1. After transfer of serum, wash the pipet with dilute  $\text{HNO}_3$  and rinse with a minimum of four portions of water and then twice with 95% ethanol. Allow pipet to drain. Apply minimum suction to the top end of the pipet for one minute to assure the absence of ethanol. The pipet should then be ready for use with the next serum sample. (NOTE: If the dilute  $\text{HNO}_3$  washings do not drain cleanly from the pipet, clean the pipet by rinsing it several times with hexane. Then repeat the cleaning and drying steps in Section III, followed by ethanol drying as described in this paragraph.

4. At the conclusion of the dilution procedures there should be:
  - a. One 50-ml volumetric flask containing a ten-fold dilution of the diluent (blank) solution, labeled "blank ten-fold dilution,"
  - b. Six 50-ml volumetric flasks containing ten-fold dilutions of each of the lithium standard diluted solutions, labeled working solutions, 0.50-, 1.00-, 1.50-, 2.00-, 2.50-, and 3.00-mmol/l of lithium, respectively, and
  - c. As many additional 50-ml volumetric flasks containing ten-fold dilutions of solutions of unknowns as there are samples to be analyzed, each labeled appropriately.

#### VI. Atomic Absorption Spectrometry (AAS) Measurement Procedure

It is assumed that the operator is fully familiar with the instrument to be used. It is not possible in this method to give detailed instructions necessary to assure instrument stability, linearity, flame conditions, etc. In general, the accuracy of the method cannot be attained unless the



instrument is in optimum operating condition and meets all the specifications set forth by the manufacturer.

1. Instrument and Electrical Adjustment. Prepare the atomic absorption spectrometer for operation according to instructions provided in the operator's manual. Place the lithium hollow cathode lamp in the lamp housing receptacle. Turn the power supply switch to "On." Select the optimum current for the lamp, and allow ample "warm-up" time for the lamp to become stable. Adjust the monochromator slit and set the wavelength selector to the lithium resonance line at 670.8 nm (6708 Å). Adjust the photomultiplier dynode voltage to give optimum current output with minimum dark current.
2. Flame Condition. Open the tank valves on the air and acetylene supplies. Adjust the secondary regulators as recommended by manufacturer. Check the burner to make sure the premixing chamber and nebulizer are clean and free of any foreign obstructions. Light the burner and adjust the air-acetylene flow rates recommended for the instrument. To stabilize the temperature of the burner head, aspirate water into the flame for 10 min before proceeding to the next step. (NOTE: A fuel-rich air-acetylene flame gives optimum sensitivity for the measurement of lithium; however, it may be difficult to obtain the precision specified in this method with a fuel-rich flame. Therefore, it is suggested that a stoichiometric or only slightly fuel-rich flame be used to obtain the highest precision for lithium in serum.)



3. Determination of Optimum Absorbance. Determine the stability and repeatability of the instrument as well as the calibration curve as follows:
  - a. Adjust the instrument to zero absorbance while nebulizing water. (NOTE: Always nebulize water when measurements of standard, blank, or unknown solutions are not being made.
  - b. Nebulize the 3.00-mmol/l lithium standard working solution and measure the absorbance.
  - c. Adjust the instrumental gain such that 90% full scale for recording instruments or 2.000 units for digital read-out instruments is observed.
  - d. Readjust the instrument to zero reading with working blank.
  - e. Nebulize the working solutions of blank, 0.50-, 1.00-, 1.50-, 2.00-, 2.50- and 3.00-mmol/l lithium standards and record the instrumental readings.
  - f. Repeat nebulization of the working blank and the 3.00 mmol/l lithium standard working solution until stable conditions are achieved.
4. Unknown Measurements.
  - a. Nebulize all the diluted unknown solutions and determine which of the two working standards bracket each of the unknowns.
  - b. For the analyses of each unknown and its two bracketing standards, reduce or increase scale expansion until the expanded absorbance for the high standard of the bracketing pair of standards reads 2.000 expanded absorbance units for digital readout instruments or approximately 90% full scale for recording instruments.



- c. Nebulize the low working standard, diluted unknown, high working standard, and diluted unknown in that order. These four measurements constitute a "set".
- d. Repeat step 4c until a valid group of 5 "sets" is obtained as illustrated in step 5 and Table 2.

Table 1

Example of Steps 3e and 4c, Section VI

Lithium Standard Diluted Solutions, mmol/l	<u>0.5001</u>	<u>1.0002</u>	<u>1.5003</u>	<u>2.0004</u>	<u>2.5005</u>	<u>3.000</u>
Expanded Absorbance Measurements						
1. Standards	0.335	0.660	1.000	1.300	1.665	2.000
2. Unknowns, A,B,C	0.473(A)		1.288(B)		1.699(C)	

5. Valid Sets of Measurements. A set of measurements is considered valid if the following conditions are met:
  - a. the two expanded absorbance values measured for the diluted unknown in a given set must not differ by more than 1%; and
  - b. the expanded absorbance values for the diluted unknown and the working standards in a given set must not differ by more than 1% from the values measured in the previous valid set.

A non-valid set must be eliminated and enough sets made to obtain a group of five valid sets. For example Set 2 in Table 2 is a valid set since the differences between the





expanded absorbance measurements for the Low Standard ( $\text{Set}_2 - \text{Set}_1 = +0.004$ ), Unknown B ( $\text{Set}_2 - \text{Set}_2 = -0.004$ ), ( $\text{Set}_2 - \text{Set}_1 = +0.006$ ) and High Standard ( $\text{Set}_2 - \text{Set}_1 = +0.002$ ) are all less than 1% of the expanded absorbance values. However, the measurements made in Set 5 are not valid because the differences between the expanded absorbance measurements for the unknowns (Unknown B set 5 minus Unknown B set 4 and Unknown B set 5 minus Unknown B set 5) are greater than the 1% limit; i.e.,  $[(1.993 - 2.032) / 2.032](100) = 1.4\%$  or  $[(2.032 - 1.995) / 1.995](100) = 1.3\%$ . Thus, Sets 1, 2, 3, 4 and 6 comprise the group of 5 valid sets and Set 5 is excluded.

Table 2

Valid Measurements for Unknown B (Steps VI-4b,c,d,5)

				EXPANDED ABSORBANCE READINGS		
Set	<u>Li, mmol/l<sup>a</sup></u>			<u>Lo Std</u> <u>1.5001</u>	<u>Unk</u> <u>B</u>	<u>Hi Std</u> <u>2.0002</u>
1	Lo Std - Unk B - Hi Std.	- Unk B -		1.536 <sup>b</sup>	1.983 <sup>b</sup> 1.987	2.000 <sup>b</sup>
2	Lo Std - Unk B - Hi Std	- Unk B		1.540	1.981 1.985	2.002
3	Lo Std - Unk B - Hi Std	- Unk B -		1.541	1.987 1.989	2.004
4	Lo Std - Unk B - Hi Std	- Unk B -		1.545	1.991 1.993	2.007
5	Lo Std - Unk B - Hi Std	- Unk B -		1.550	2.032 1.995	2.009
6	Lo Std - Unk B - Hi Std	- Unk B -		1.556	1.997 1.999	2.011

a Calculate and report Li, mmol/l to four decimal places.

b Record and report all expanded absorbance values with four figures on data sheets provided.



6. Data Recording and Calculations. Please record the lithium standard diluted solution concentrations in mmol/l of Li to four decimal places and the expanded absorbance readings to four figures on the attached form labeled "Data Sheet" and as illustrated in Tables 1 and 2. No calculations are required for this round robin test. Data evaluation will be performed at NBS and equations for the calculations which give the best expanded absorbance values and subsequent mmol/l of Li will be determined.

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1. Pybus, J., Bowers, G.N. Jr., "Measurement of Serum Lithium by Atomic Absorption Spectroscopy," Clin. Chem. 16, 139-143 (1970).
  2. Office of Standard Reference Materials, The National Bureau of Standards, Washington, D.C. 20234.
  3. Reagent Chemicals, American Chemical Society Specifications ACS Publications, Washington, D.C. (1968).
  4. Hughes, J.C., "Testing of Glass Volumetric Apparatus," NBS Handbook 77, Volume III, 726/1-10, (1961). "Precision Measurement and Calibration," A compilation by S.F. Booth of selected papers on Optics, Metrology and Radiation.



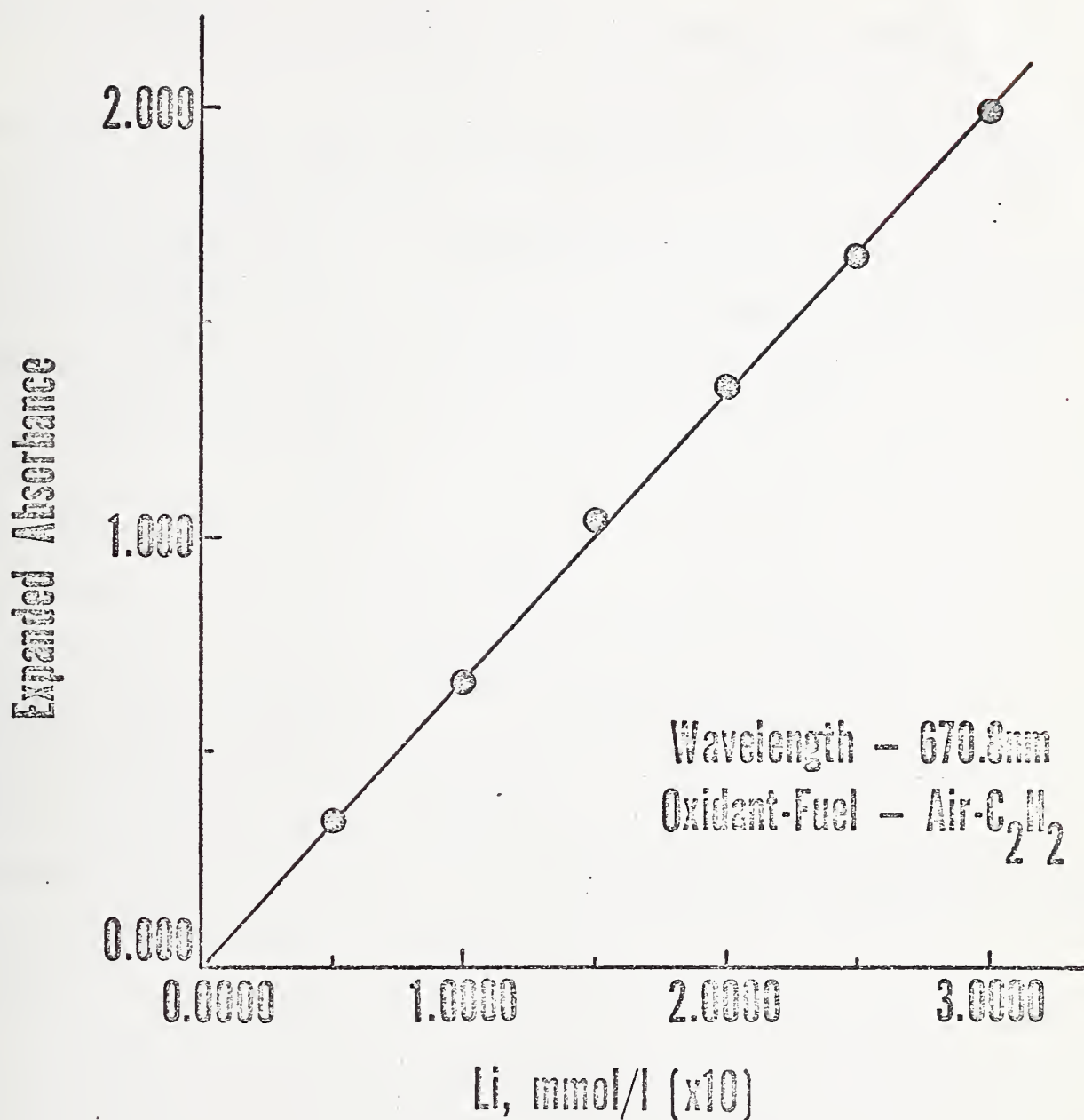


Figure 1. Typical Calibration Curve for the Atomic Absorption Spectrometric Determination of Lithium. Data Given in This Graph Kindly Supplied by Mr. Michael Epstein and Mr. Theodore Rains, NBS.



NBS - Electrolytes in Serum - Clinical Reference Method

Lithium

Laboratory \_\_\_\_\_ Analyst \_\_\_\_\_

Exercise No. \_\_\_\_\_ RRI \_\_\_\_\_

Date Samples Received \_\_\_\_\_ Dates Analyzed (1) \_\_\_\_\_ (2) \_\_\_\_\_

Instrument Manufacturer \_\_\_\_\_ Model \_\_\_\_\_

Wavelength \_\_\_\_\_ nm Slit Width \_\_\_\_\_  $\mu$ m

Type Hollow Cathode Lamp \_\_\_\_\_

Current \_\_\_\_\_ mA

Burner Type \_\_\_\_\_

Oxidant \_\_\_\_\_ Flow rate \_\_\_\_\_ l/min

Fuel \_\_\_\_\_ Flow rate \_\_\_\_\_ l/min

Instrument Time Constant \_\_\_\_\_ s Scale Expansion \_\_\_\_\_

Recorder Time Constant \_\_\_\_\_ s

Readout: Recorder \_\_\_\_\_, Digital \_\_\_\_\_, Other \_\_\_\_\_

Laboratory Temperature \_\_\_\_\_  $^{\circ}$ C to \_\_\_\_\_  $^{\circ}$ C (Variation during Round Robin

Background Correction? \_\_\_\_\_ How? \_\_\_\_\_

Comments: \_\_\_\_\_

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DATA SHEET

\_\_\_\_ Round Robin \_\_\_\_

Date: \_\_\_\_\_

EXPANDED ABSORBANCE

	Li, mmol/l	Lo Std	Unknown	Hi Std	Valid	Remark
Set <sup>a</sup>		_____		_____		
1		_____	_____ _____	_____		
2		_____	_____ _____	_____		
3		_____	_____ _____	_____		
4		_____	_____ _____	_____		
5		_____	_____ _____	_____		
6		_____	_____ _____	_____		
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<sup>a</sup> Only 5 valid sets needed.

